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13. ABSTRACT ***(Maximum 200 Words)***

The hypothesis to be tested was that an increase in 12-lipoxygenase activity in prostate cancer cells would increase their angiogenic potential, either directly through the direct metabolite 12(S)-HETE or indirectly, through stimulation of angiogenic factors. The preliminary data regarding increased vascularization by 12-LOX transfectant PC-3 cells was confirmed in two additional PC-3 transfectants. In addition, we were able to demonstrate that 12(S)-HETE does stimulate endothelial cell migration. This stimulated migration occurred through activation of MAP kinase. We also determined that 12-LOX transfected cells secreted increased amounts of VEGF but not bFGF or IL8. Further studies demonstrated that 12(S)-HETE activated the VEGF promoter activity and we also confirmed increased VEGF mRNA in 12-LOX transfectants by Northern blot. The role of increased VEGF production in 12-LOX transfectants was confirmed using a neutralizing antibody. The first four technical objectives have been met and work continues on technical objective five and six.

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OUTLINE

INTRODUCTION

The Award DAMD17-98-1-8502 studies the role of bioactive lipids in prostate cancer angiogenesis. The specific purpose of this research is to investigate whether the expression of 12-lipoxygenase (LOX) and subsequent synthesis of 12(S)-HETE modulate angiogenesis in prostate cancer. The effect of 12-LOX and 12(S)-HETE on endothelial cell migration, expression of putative angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), and angiogenesis in tumor and in other animal models will be addressed in this award. Knowledge obtained from these endeavors potentially can be translated into novel therapeutic approach for the treatment of human prostate cancer.

BODY OF REPORT

List of Technical Objectives

1. Corroborate the preliminary data regarding increased vascularization of tumors formed by 12-LOX transfected PC3 cells vs. neocontrols in two additional PC3 transfectant clones and determine the vessel density, proliferation index, and apoptotic index in these tumors.
2. Determine in vitro using endothelial cell migration assay that the observed increase in endothelial cell migration to PC3 12-LOX transfectants is due wholly or partly to increased 12-LOX activity.
3. If the increased endothelial cell migration cannot be accounted for in total by increased 12(S)-HETE production, then we will determine if there is increased expression of putative angiogenic factors or decreased levels of natural angiogenesis inhibitors in 12-LOX transfectants. If such effect is positive and if enhanced endothelial cell migration cannot be accounted for in total by 12(S)-HETE then, we will proceed to technical objective 4.
4. Determine in vitro if exogenous 12(S)-HETE can enhance the expression of the angiogenic factor(s) or reduce the levels of endogenous inhibitor(s) identified in the previous Objective.
5. Determine if 12(S)-HETE has a direct effect on angiogenesis *in vivo* using the CAM assay and the Matrigel implantation assay.
6. Determine whether a natural bioactive lipid i.e., 13(S)-HODE can antagonize the effects of 12(S)-HETE in specific aims two through five above.

In Phase II, we will do 12-LOX inhibition study to see whether the increased angiogenesis in 12-LOX derived tumors can be inhibited by BHPP. We will further study the role of 12(S)-HETE in vascularization by using a construct with controlled expression of 12-LOX. Finally, we will study whether PCa angiogenesis can be inhibited by boosting 13(S)-HODE synthesis (Please see the Statement of Work for details).

Research Progress

Objective 1. Corroborate the preliminary data regarding the increased vascularization of tumors formed by 12-LOX transfected PC3 cells vs. neo-controls in two additional PC3 transfectant clones.

This technical objective has been achieved. Specifically, we have confirmed the preliminary data regarding the increased vascularization in tumors formed by 12-LOX transfected PC3 cells with three independent clones, nL-2, nL-8, and nL-12. The increased tumor growth, increased angiogenesis and decreased necrosis in tumors derived from 12-LOX transfected PC3 cells were reported in detail in our publication in Cancer Research (Nie et al., 1998. Appendix A).

Objective 2. Determine the observed increase in endothelial migratory response to PC3 12-LOX transfectants is due wholly or in part to increased 12-lipoxygenase activity.

This technical objective has been achieved. We found that 12(S)-HETE can stimulate endothelial cell migration as reported in our publication in Cancer Research (Nie et al., 1998). Further, inhibition of the activation of P42/44 MAP kinase by PD98059 abrogated 12(S)-HETE stimulated endothelial cell migration (**Figure 1**). The data suggest 12-LOX and 12(S)-HETE can stimulate angiogenesis by stimulating endothelial cell migration in a P42/44 MAP kinase dependent way.

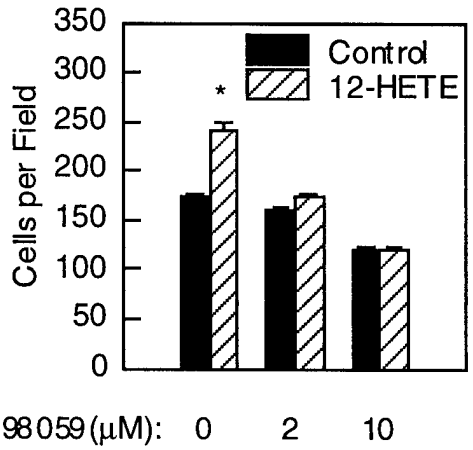

Figure 1. MAP Kinase Dependent Stimulation of Endothelial Cell Migration by 12(S)-HETE. *, $P < 0.05$ when compared to its control.

To study whether 12-LOX transfected PC3 cells can secrete more protein factors, in addition to bioactive lipid 12(S)-HETE, to stimulate endothelial cell migration, the spent culture media were concentrated 8-fold using centricon-10 to eliminate small molecules and examined for the ability of the concentrates to stimulate endothelial cell migration. As shown in **Figure 2**, the media concentrate from neo-control cells have angiogenic factors to stimulate endothelial cell migration. 12-LOX transfected PC3 cells secreted more angiogenic factors to stimulate endothelial cell migration. Moreover, the increased stimulation of endothelial cell migration can be blocked by neutralizing antibody against VEGF, but not bFGF or IL8, suggesting that 12-LOX transfected PC3 cells can produce more VEGF to stimulate angiogenesis. The finding led us to address the Technical Objective 3.

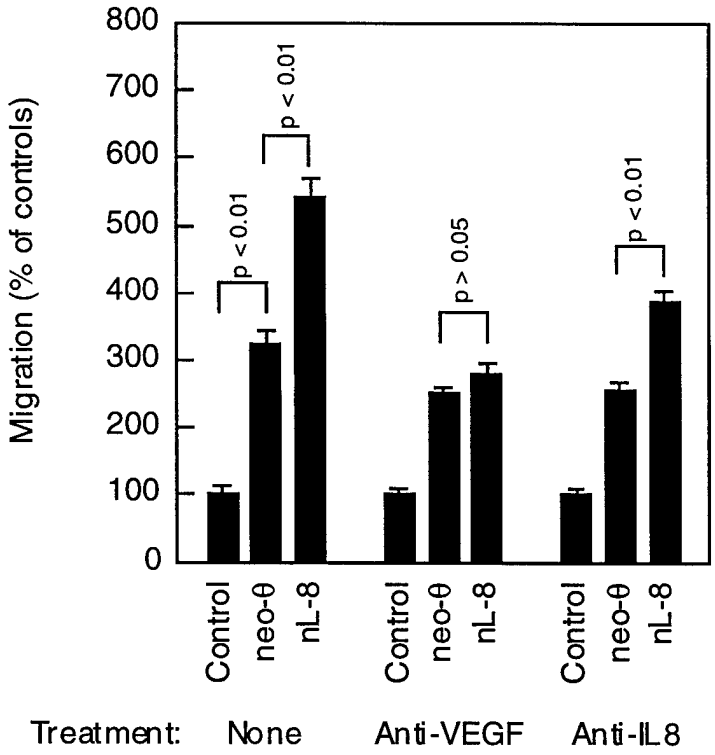

Figure 2. 12-LOX Transfected PC3 Cells Produced More VEGF to Stimulate Endothelial Cell Migration. The spent culture media were concentrated and then were added to EBM-2 media in the low chamber. The numbers of HUVEC migrated after overnight incubation were counted in a blind approach. For control, concentrated unused RPMI-media were added to EBM-2 in the low chamber.

Objective 3. Determine whether there is increased expression of putative angiogenic factors or decreased levels of natural angiogenesis inhibitors in 12-LOX transfectants.

Since we found that 12-LOX-transfected PC3 cells produce more high molecular weight angiogenic factors to stimulate endothelial cell migration than did neo-controls and that this increased angiogenicity of 12-LOX transfected PC-3 cells can be neutralized by antibody against VEGF, but not bFGF or IL-8, next we measured the levels of VEGF, IL8, and bFGF. It was found that 12-LOX transfected PC3 cells secrete two- to three-fold more VEGF in the culture media than did neo-control as measured by ELISA (**Figure 3**). Furthermore, NDGA, a general LOX inhibitor, and baicalein, a specific 12-LOX inhibitor, reduced VEGF expression in 12-LOX transfected PC3 cells (**Figure 3**). We also measured the level of IL-8 and bFGF in 12-LOX transfected PC3 cells and found no change in IL-8 or bFGF secretion in 12-LOX transfected PC3 cells as compared to neo-controls.

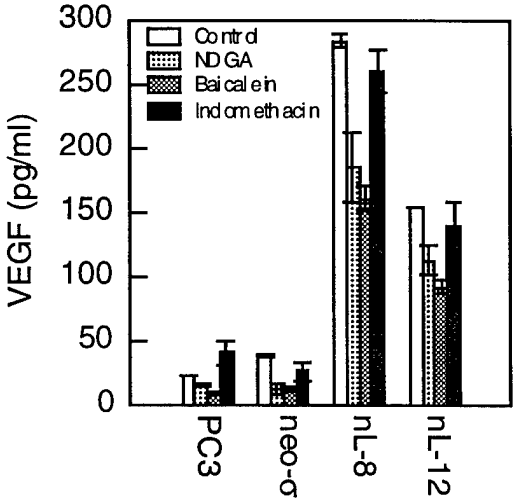

Figure 3. Increased VEGF Expression in 12-LOX Transfected PC3 Cells and The Effect of 12-LOX Inhibitors. VEGF levels in conditioned media were measured by ELISA. Note, NDGA, a general LOX inhibitor, reduced VEGF expression. Baicalein,a specific 12-LOX inhibitor, also significantly reduced VEGF expression while a cyclooxygenase inhibitor, indomethacin, had no effect.

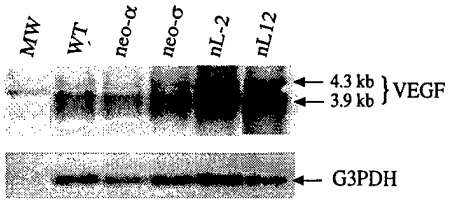

Figure 4. Northern Blot Analysis of VEGF mRNA Levels. Poly(A)+RNA were isolated and the 12-LOX mRNA levels were analyzed with labeled VEGF cDNA. G3PDH probe was used for the control.

Northern blot analysis revealed there is an increase of 12-LOX mRNA levels in 12-LOX transfectants (**Figure 4**). To further study whether there is an increased VEGF expression at the transcriptional level, VEGF promoter -1174/+54 luciferase construct was introduced into nL8 and nL-12. It was found that there is 20-fold more promoter activity in 12-LOX transfected PC3 cells when compared to their neo-control (**Figure 5**). The data suggest that 12-LOX, possibly through its product 12(S)-HETE, stimulates VEGF promoter activity.

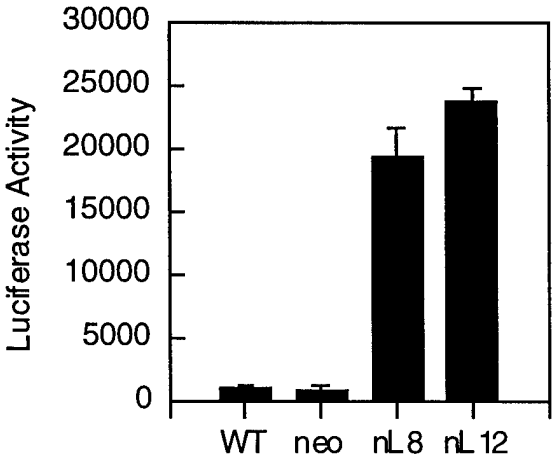

Figure 5. Increased VEGF Promoter Activity in 12-LOX Transfected PC3 Cells. Cells were transfected with a VEGF promoter luciferase construct, p -1176/+54. Cells were harvested 24 hours after transfection and then the luciferase activity was measured by scintillation counting using a kit from Promega Corp. (Madison, WI).

Technical Objective 4. Determine in vitro if exogenous 12(S)-HETE can enhance the expression of the angiogenic factor(s) or reduce the levels of endogenous inhibitor(s) identified in previous Objective.

This technical objective has been partially met. We have studied whether exogenous 12(S)-HETE can increase VEGF expression. As shown in **Figure 6**, 12(S)-HETE treatment of PC3 cells stimulated VEGF production in a dose-dependent manner. Further, 12(S)-HETE dose-depended stimulated VEGF promoter activity (**Figure 7**). Currently, we are investigating the signaling pathway by which 12(S)-HETE stimulates VEGF promoter activity in PC3 cells as well as in DU145 and LnCAP cells.

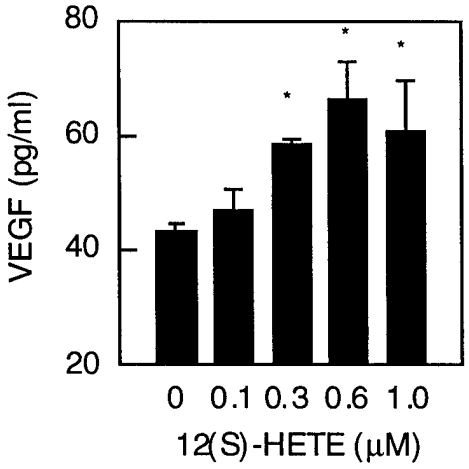

Figure 6. Stimulation of VEGF secretion in PC3 cells by 12(S)-HETE. Semi-confluent PC3 cells were incubated in RPMI-0.2%BSA and treated with graded dose of 12(S)-HETE. One day after treatment, the media were collected and VEGF levels in the media were measured using an ELISA kit from R&D Systems (Minneapolis, MN). *, $P < 0.05$ when compared to the control.

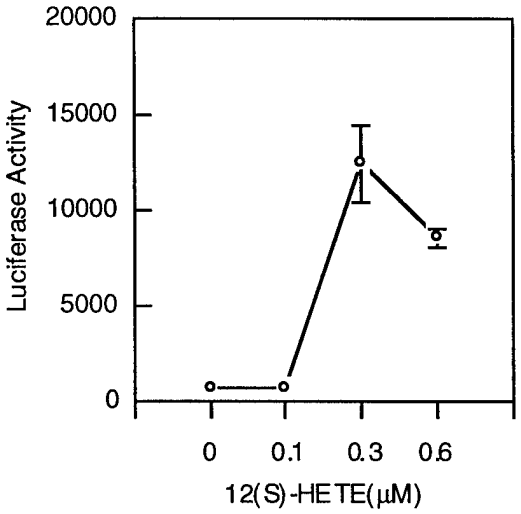

Figure 7. Stimulation of VEGF Promoter Activity by 12(S)-HETE. PC3 cells were transfected with a VEGF promoter luciferase construct, p -1176/+54 and then treated with graded levels of 12(S)-HETE. Cells were harvested 18 hours after treatment and then the luciferase activity was measured by scintillation counting using a kit from Promega Corp. (Madison, WI).

Technical Objective 5. Determine if 12(S)-HETE has a direct effect on angiogenesis in vivo using the CAM assay and the Matrigel implantation assay.

The studies proposed in this Technical Objective are ongoing.

Technical Objective 6. Determine whether a natural bioactive lipid i.e., 13(S)-HODE can antagonize the effects of 12(S)-HETE in technical objectives two through five above.

The studies proposed in this technical objective are ongoing.

Discussion

The results obtained so far support our original hypothesis that the increased expression of 12-LOX and, thereby, its metabolite 12(S)-HETE, during prostate cancer progression, represents a novel and previously unrecognized angiogenic switch. First, we found that 12-LOX, when overexpressed in PC3 cells, stimulated prostate tumor growth and angiogenesis. Second, we found that 12(S)-HETE, the arachidonate product of 12-LOX activity, directly stimulates endothelial cell migration and this may involve the activation of p42/44 MAP kinase. Third, we

also found, in addition of 12(S)-HETE, 12-LOX in PC3 cells can enhance the secretion of VEGF to stimulate angiogenesis. The observation was further confirmed by ELISA measurement of VEGF levels, Northern Blot and VEGF promoter activity analysis. Forth, we found that exogenously added 12(S)-HETE was capable to stimulate VEGF expression as well as VEGF promoter activity. These observations, made in the first year of Award# DAMD17-98-1-8502, support our hypothesis that 12-LOX played a contributory role in prostate cancer angiogenesis on one hand. On the other hand, these observations have significantly furthered our understanding of prostate cancer angiogenesis in general and lipid regulation of angiogenesis in particular.

KEY RESEARCH ACCOMPLISHMENTS

- The ability of 12-LOX to increase prostate cancer angiogenesis was confirmed.
- 12(S)-HETE, the arachidonate product of 12-LOX, was found to activate p42/44 MAP kinase and stimulate endothelial cell migration.
- Increased VEGF secretion in 12-LOX transfected PC3 cells was demonstrated using ELISA.
- Increased VEGF secretion in 12-LOX transfected PC3 cells was reduced by 12-LOX inhibition by NDGA or baicalein.
- Increased VEGF expression was demonstrated in 12-LOX transfected PC3 cells by Northern Blot and VEGF promoter luciferase activity assay.
- The role of increased VEGF expression in 12-LOX transfected PC3 cells was demonstrated functionally by the effect of VEGF neutralizing antibody on the stimulated endothelial cell migration by 12-LOX transfected PC3 cells.
- 12(S)-HETE stimulated VEGF secretion and VEGF promoter activity in PC3 cells.
- The regulation of VEGF expression by 12-LOX and 12(S)-HETE, as found in the first year of this award, is a novel finding and will have significant implications in other types of cancers and other diseases in which uncontrolled angiogenesis play a prominent role.

REPORTABLE OUTCOMES

- Research article published.
  Nie, D., G. G. Hillman, T. Geddes, K. Tang, C. Pierson, D. J. Grignon and K. V. Honn. 1998. Platelet-type 12-lipoxygenase in a human prostate carcinoma stimulates angiogenesis and tumor growth. Cancer Research 58: 4047-4051.
- Abstract published.
  Nie, D., J. A. Nemeth, M.L. Cher, Y. Chen, U. Barrosso, and K.V. Honn. 1999. Inhibition of prostate cancer cells by a novel 12-lipoxygenase inhibitor in a human orthotopic bone metastasis model. Proc. Amer. Assoc. Cancer Res. 40: 126.
- Abstract published.
  Nie, D., Y. Chen, K. Tang, K. Hanna, J. Nemeth, G.G. Hilman, M. Cher, D. Grignon, and K.V. Honn. 1999. Arachidonate 12-lipoxygenase enhances the metastatic potential of human prostate cancer cells. Proc. Amer. Assoc. Cancer Res. 40: 198.
- Abstract in-press.

Nie, D., Y. Chen, K. Tang, G.G. Hillman, D. Grignon, and K.V. Honn. 1999. Arachidonate 12-lipoxygenase stimulates angiogenesis by up-regulation of vascular endothelial growth factor expression. Prostaglandins and Other Lipid Mediators (In Press).

- Presentation.
  Nie, D., J. A. Nemeth, M.L. Cher, Y. Chen, U. Barrosso, and K.V. Honn. "Inhibition of prostate cancer cells by a novel 12-lipoxygenase inhibitor in a human orthotopic bone metastasis model." Poster Presentation at the American Association for Cancer Research 1999 Annual Meeting, Philadelphia, PA, April 10-14, 1999.

- Presentation.
  Nie, D., Y. Chen, K. Tang, K. Hanna, J. Nemeth, G.G. Hilman, M. Cher, D. Grignon, and K.V. Honn. "Arachidonate 12-lipoxygenase enhances the metastatic potential of human prostate cancer cells." Mini-Symposium Presentation at the American Association for Cancer Research 1999 Annual Meeting, Philadelphia, PA, April 10-14, 1999.

- Presentation.
  Nie, D., Y. Chen, K. Tang, G.G. Hillman, D. Grignon, and K.V. Honn.. "Arachidonate 12-lipoxygenase stimulates angiogenesis by up-regulation of vascular endothelial growth factor expression." Oral Presentation Selected by the 6th International Conference on Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Related Diseases, Boston, MA, Sept. 12 -15, 1999.

- Patents applied: None.

- Degrees obtained that are supported by this award: None.

- Development of cell lines, tissue or serum repositories: None

- Funding applied or obtained: Yes
  Based on the above-described findings, PI was recognized by a CaPCURE award in 1999, in the amount of $75,000, to develop prostate-targeting 12-LOX inhibitors for the treatment of prostate cancer.

CONCLUSIONS:

In the first year of this award, we have made the following progress: First, we confirmed that 12-LOX, when overexpressed in PC3 cells, stimulated prostate tumor growth and angiogenesis. The results, together with our previous observation that 12-LOX expression is positively correlated with tumor stage, implicate the important role of 12-LOX in prostate angiogenesis and suggest that inhibition of 12-LOX activity be a novel target for developing anti-angiogenesis therapy for the treatment of prostate cancer.

Second, we found that 12(S)-HETE, the arachidonate product of 12-LOX activity, directly stimulates endothelial cell migration and this may involve the activation of p42/44 MAP kinase. The pro-angiogenic activity of 12(S)-HETE as found here has significant implications. It explains the increased angiogenic potentials of 12-LOX transfected PC3 cells. On the other hand, since platelets are rich source for 12(S)-HETE, our findings implicate the potential pro-involvement of platelets in a number of pathological settings in which angiogenesis plays an important role.

Third, we also found, in addition of 12(S)-HETE, 12-LOX in PC3 cells can enhance the secretion of VEGF to stimulate angiogenesis. Further, we found that exogenously added 12(S)-

HETE was capable to stimulate VEGF expression as well as VEGF promoter activity. The observation was further confirmed by ELISA measurement of VEGF levels, Northern Blot and VEGF promoter activity analysis. The novel link between 12-LOX and VEGF established here provides the first example of the regulation of VEGF, a putative angiogenic factor, by a lipid molecule and also provides a new dimension of the biological activity of 12-LOX and its eicosanoid 12(S)-HETE.

In summary, the above-described research progress, made in the first year of Award# DAMD17-98-1-8502, support our hypothesis that 12-LOX played a contributory role in prostate cancer angiogenesis. On one hand, the study identified 12-LOX as an important molecule controlling the angiogenic potential of prostate cancer, suggesting 12-LOX as a novel target for developing anti-angiogenesis therapy for the treatment of prostate cancer. On the other hand, these observations have provided significant insights into our understanding of the regulation of VEGF expression. This knowledge also may be applied to our understanding of angiogenesis in other types of tumor.

REFERENCES

Nie D, Hillman GG, Geddes T, Tang K, Pierson C, Grignon DJ, and Honn KV. (1998) Platelet-type 12-lipoxygenase in a human prostate carcinoma stimulates angiogenesis and tumor growth. Cancer Res 58: 4047-4051.

APPENDIX

1). Research article published.
Nie, D., G. G. Hillman, T. Geddes, K. Tang, C. Pierson, D. J. Grignon and K. V. Honn. 1998. Platelet-type 12-lipoxygenase in a human prostate carcinoma stimulates angiogenesis and tumor growth. Cancer Research 58: 4047-4051.

2) Abstract published.
Nie, D., J. A. Nemeth, M.L. Cher, Y. Chen, U. Barrosso, and K.V. Honn. 1999. Inhibition of prostate cancer cells by a novel 12-lipoxygenase inhibitor in a human orthotopic bone metastasis model. Proc. Amer. Assoc. Cancer Res. 40: 126.

3) Abstract published.
Nie, D., Y. Chen, K. Tang, K. Hanna, J. Nemeth, G.G. Hilman, M. Cher, D. Grignon, and K.V. Honn. 1999. Arachidonate 12-lipoxygenase enhances the metastatic potential of human prostate cancer cells. Proc. Amer. Assoc. Cancer Res. 40: 198.

4) Abstract in-press.
Nie, D., Y. Chen, K. Tang, G.G. Hillman, D. Grignon, and K.V. Honn. 1999. Arachidonate 12-lipoxygenase stimulates angiogenesis by up-regulation of vascular endothelial growth factor expression. Prostaglandins and Other Lipid Mediators (In Press).

[CANCER RESEARCH 58, 4047–4051, September 15, 1998]

Advances in Brief

Platelet-Type 12-Lipoxygenase in a Human Prostate Carcinoma Stimulates Angiogenesis and Tumor Growth[1]

Daotai Nie, Gilda G. Hillman, Timothy Geddes, Keqin Tang, Christopher Pierson, David J. Grignon, and Kenneth V. Honn[2]

Departments of Radiation Oncology [D. N., T. G., K. T., K. V. H.], Urology [G. G. H.], and Pathology [C. P., D. J. G., K. V. H.], Wayne State University School of Medicine, Detroit, Michigan 48202

Abstract

Previously, we found a positive correlation between the expression of platelet-type 12-lipoxygenase (12-LOX) and the progression of human prostate adenocarcinoma (PCa; Gao *et al.*, Urology, *46:* 227–237, 1995). To determine the role of 12-LOX in PCa progression, we generated stable 12-LOX-transfected PC3 cells, which synthesize high levels of 12-LOX protein and 12(*S*)-hydroxyeicosatetraenoic acid metabolite. *In vitro*, 12-LOX-transfected PC3 cells demonstrated a proliferation rate similar to neo controls. However, following s.c. injection into athymic nude mice, 12-LOX-transfected PC3 cells formed larger tumors than did the controls. Decreased necrosis and increased vascularization were observed in the tumors from 12-LOX-transfected PC3 cells. Both endothelial cell migration and Matrigel implantation assays indicate that 12-LOX-transfected PC3 cells were more angiogenic than their neo controls. These data indicate that 12-LOX stimulates human PCa tumor growth by a novel angiogenic mechanism.

Introduction

The growth and metastasis of solid tumors are dependent upon the ability of tumor cells to induce angiogenesis (1). Angiogenesis, the formation of new blood vessels from preexisting ones, involves endothelial cell proliferation, motility, and differentiation. Tumor cells can secrete a variety of angiogenic factors, such as basic fibroblast growth factor and vascular endothelial growth factor, to stimulate angiogenesis (2). Tumor cells also produce angiogenesis inhibitors such as thrombospondin and angiostatin to control angiogenesis (2). The balance between angiogenesis stimulators and inhibitors determines the angiogenicity of tumor cells (2). In human PCa,[3] the level of vascularization positively correlates with tumor stage (3–5). Inhibition of angiogenesis by linomide or TNP-470 potently inhibits PCa growth and metastasis by causing necrosis and apoptosis in tumors (6, 7). Although various potential angiogenesis factors have been identified in prostate cancer (8), it is still unclear by which process PCa cells become angiogenic. We have previously detected the expression of platelet-type 12-LOX in human PCa and demonstrated a correlation between 12-LOX mRNA expression and pathological stage (9). Platelet-type 12-LOX uses only arachidonic acid as substrate and forms 12(*S*)-HETE exclusively (10). Here, we have examined the function of 12-LOX on PCa tumor growth. Our data demonstrate that 12-LOX has no detectable effect on PCa cell growth *in vitro* but stimulates PCa tumor growth *in vivo*. This effect of 12-LOX on tumor growth is closely related to increased angiogenesis. Both *in vitro* and *in vivo* angiogenesis assays suggest that PCa cells expressing high levels of 12-LOX are more angiogenic than those expressing no or low levels of 12-LOX. Our results provide a novel function for platelet-type 12-LOX in PCa progression.

Materials and Methods

Cell Culture. Rat angiogenic endothelial cell line RV-ECT (a gift from Dr. Clement Diglio, Department of Pathology, Wayne State University) was maintained in DMEM with 10% FBS (11). The cells were used between passage numbers 29 and 34. The human prostate carcinoma cell line PC3 was originally purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 with 10% FBS. All culture reagents were purchased from Life Technologies, Inc.

Stable Transfection of PC3 Cells and Characterization. Passage 28 PC3 cells were cotransfected using a Lipofectin reagent (Life Technologies, Inc.) with a pCMV-platelet-type 12-LOX construct (a gift from Dr. Collin Funk, Center for Experimental Therapeutics, University of Pennsylvania; Ref. 10), and pCMV-*neo*, which encodes a neomycin-resistant protein. PC3 cells transfected with pCMV-*neo* were used as controls. Transfectants were selected using 1 mg/ml geneticin (G418) in RPMI with 10% FBS and then cloned using a limiting dilution method in 96-well plates. The cloned transfectants were propagated and characterized for 12-LOX mRNA expression by Northern blot and 12-LOX protein expression by Western blot. Human epidermoid carcinoma A431 cells that express 12-LOX (12) were used as a positive control. The probe used in Northern blot was the 12-LOX cDNA from pCMV 12-LOX construct. Rabbit 12-LOX polyclonal antibody used in Western blot was purchased from Oxford Biomedical Inc. (Oxford, MI). Actin antibody was from Amersham (Arlington Heights, IL). The synthesis of 12(*S*)-HETE by 12-LOX transfectants was determined using a RIA kit from Perspective Diagnostics (Cambridge, MA) according to the manufacturer's instructions.

***In Vitro* Proliferation Assay.** To study the growth kinetics of PC3 transfectants in culture, 2×10^3 cells per well were seeded in 96-well culture plate. The number of viable cells at intervals of 48 h was assessed using an MTS cell proliferation assay kit (Promega Corp, Madison, MI). The $A_{490\ nm}$ readings 2–3 h after plating were used as baselines. The number of cells was expressed as the percentage of increase from the $A_{490\ nm}$ baselines.

Animal Model and Histochemical Studies. A total of 4×10^6 12-LOX-transfected PC3 cells or neo control cells in 200 μl of HBSS were injected s.c. into the right flank of 4–6-week-old male BALB/c nude mice (obtained from University of South Florida, Tampa, FL). The resulting tumors were measured using a vernier caliper, and tumor volume was calculated using the formula: $(\text{width})^2 \times \text{length} \times 0.5$ (7). Six to 7 weeks after injection, mice were sacrificed, and the tumors were resected and photographed under an SP SZ-4060 stereomicroscope (Olympus America, Melville, NY). Tumors were fixed in 10% neutral buffered formalin and embedded in paraffin, and sections (5 μm) were prepared for histology staining. Sections were stained with H&E to examine the presence of necrosis. The assessment of tumor necrotic area was performed for a total of 10 HPFs per tumor using a double-blind approach.

CD31 staining was used to assess tumor vascularization. Immunohistochemical staining for CD31 (DAKO Corp.; dilution, 1:20) was performed

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[3] The abbreviations used are: PCa, prostate adenocarcinoma; 12-LOX, 12-lipoxygenase; 12(*S*)-HETE, 12(*S*)-hydroxyeicosatetraenoic acid; RV-ECT, rat vascular endothelial cells-tube forming; FBS, fetal bovine serum; HPF, high-power field.

using a standard avidin-biotin complex-immunoperoxidase procedure. The slides were counterstained with hematoxylin. The vascularity was assessed qualitatively on the basis of overall vessel organization and quantitatively by microvessel density. A total of 10 fields per tumor were evaluated for both microvessel density and vessel organization. The microvessel density was indicated by the average number of vessels crossing an arbitrary line across one HPF field. The rating of vessel organization was performed according to the following scale: 0, disorganized, staining randomly distributed; 1, intermediate, vessel-like structures formed; and 2, highly organized, vessels structured and organized.

Endothelial Cell Migration Assay. For the cell migration assay, RV-ECT endothelial cells were harvested by trypsinization and resuspended in RPMI with 10% FBS, and 5×10^5 cells in 0.5 ml were plated on the top chamber of a modified Boyden chamber (Becton Dickinson, Bedford, MA). Then, 1 ml of RPMI-10% FBS medium conditioned from PC3 or various transfectant cultures or fresh medium with 12(*S*)-HETE was added in triplicate into the lower chamber. After 4 h of incubation, the cells on the top side of the transwell membrane were removed with cotton swabs. The membrane was then cut out, fixed in a quick-fix solution, double-stained, and mounted for observation and counting. Usually, 12 fields (×100) representing two perpendicular cross-lines of each membrane were counted.

Matrigel Implantation Assay for Tumor Cell-induced Angiogenesis. The Matrgel implantation assay was performed as described by Ito *et al.* (13) with the following modifications. Matrigel (Becton Dickinson, Bedford, MA; 0.4 ml premixed with 2×10^6 PC3 12-LOX transfectant or neo control cells) was injected s.c. into nude mice (four mice per group). Mice were sacrificed 12 days after injection and dissected to expose the implants for recording.

Results

Generation of PC3 Transfectants That Constitutively Synthesize 12-LOX and 12(*S*)-HETE. To determine the function of 12-LOX in PCa progression, PC3 cells were transfected with a platelet-type 12-LOX cDNA construct. Stable transfectants were cloned and named the nL series. Several stable transfectants (neo series) isolated from PC3 cells transfected with pCMV-*neo* were used as controls. Northern blot analyses of transfectant clones show that the levels of 12-LOX mRNA were increased in various nL clones, compared to the neo controls or wild-type PC3 (Fig. 1*A*). The 12-LOX mRNA levels in various nL clones were higher than in A431, a cell line that constitutively expresses 12-LOX (12). 12-LOX-transfected PC3 cells also had higher levels of 12-LOX protein than neo controls or wild-type PC3, as revealed by Western blot analysis (Fig. 1*B*). Among the various clones analyzed, nL-2, nL-8, nL-11, and nL-12 expressed 12-LOX at the highest levels. We also found that 12-LOX-transfected PC3 clones nL-2, nL-8, and nL-12 synthesized 6–10-fold more 12(*S*)-HETE than the neo control or wild-type PC3 cells (Fig. 1*C*), indicating that 12(*S*)-HETE biosynthesis was greatly enhanced in 12-LOX-transfected PC3 cells.

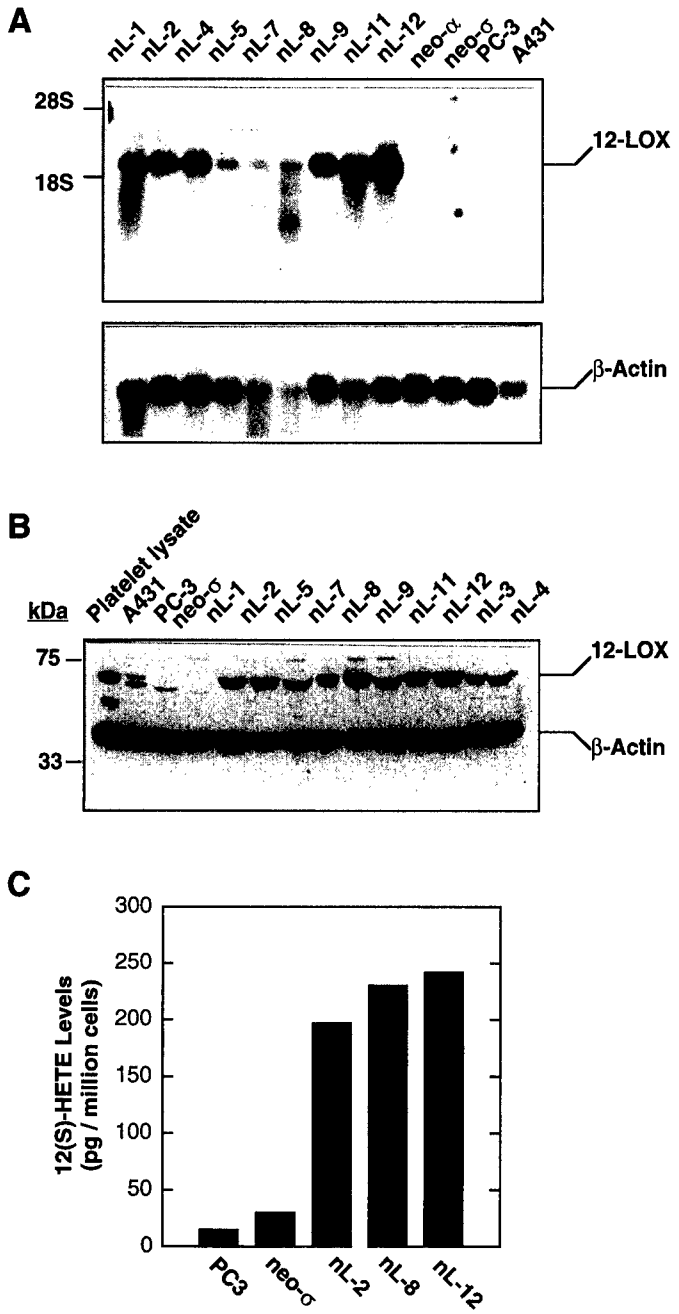

Fig. 1. Generation of PC3 transfectants synthesizing high levels of 12-LOX and 12(*S*)-HETE. The transfection of PC3 cells and the cloning of stable transfectants were performed as described in "Materials and Methods." *A*, Northern blot analysis of 12-LOX mRNA levels in various clones of PC3 12-LOX transfectants. *Top*, blot probed with 12-LOX cDNA; *bottom*, blot probed with actin cDNA as the loading control. *B*, Western blot analysis of 12-LOX protein expression in various clones of PC3 12-LOX transfectants. The blot was probed with a 12-LOX polyclonal antibody and actin antibody. *C*, 12(*S*)-HETE levels in various 12-LOX transfectants. The levels of 12(*S*)-HETE in total cell lysates were measured using RIA and were normalized to cell number and expressed as pg of 12(*S*)-HETE/1 × 10^6 cells.

12-LOX Transfectants Have an *in Vivo* but not an *in Vitro* Growth Advantage. *In vitro*, the growth rates of several 12-LOX transfectant clones were similar to those of neo controls and wild-type PC3 cells (Fig. 2*A*), with an approximate doubling time of 36 h. However, following s. c. injection into nude mice, 12-LOX-transfected PC3 cells (nL-2 and nL-12) grew faster and formed larger tumors than did neo controls (neo-σ and neo-α; Fig. 2*B*). As shown in Fig. 2*C*, tumors derived from 12-LOX-transfected PC3 cells were larger than those obtained from neo controls, indicating that 12-LOX-transfected PC3 cells had an *in vivo* growth advantage compared to neo controls or wild-type PC3 cells. Similar results were obtained with an additional 12-LOX transfectant clone tested (nL-8; data not shown). Assessment of tumor necrosis from H&E-stained tumor sections revealed that tumor necrosis was significantly reduced in the tumors derived from 12-LOX-transfected PC3 cells ($P < 0.05$ by Student's *t* test), whereas 12.1% of tumor area of neo-σ tumors were necrotic ($n = 7$; range, 5–35%), only 1.9% of tumor area in the nL-12 clone was necrotic ($n = 8$; range, 0–10%). A significant decrease in tumor necrosis was also observed in the tumors derived from 12-LOX transfectants nL-2 and nL-8, compared to neo-α (data not shown), suggesting that the increased tumor growth by 12-LOX transfectants is mainly due to the reduction of tumor necrosis.

Increased Angiogenesis in the Tumors from 12-LOX Transfectants. Because angiogenesis plays an important role in tumor growth by influencing tumor necrosis and apoptosis (2), we studied whether the increased tumor growth by 12-LOX transfectant is angiogenesis

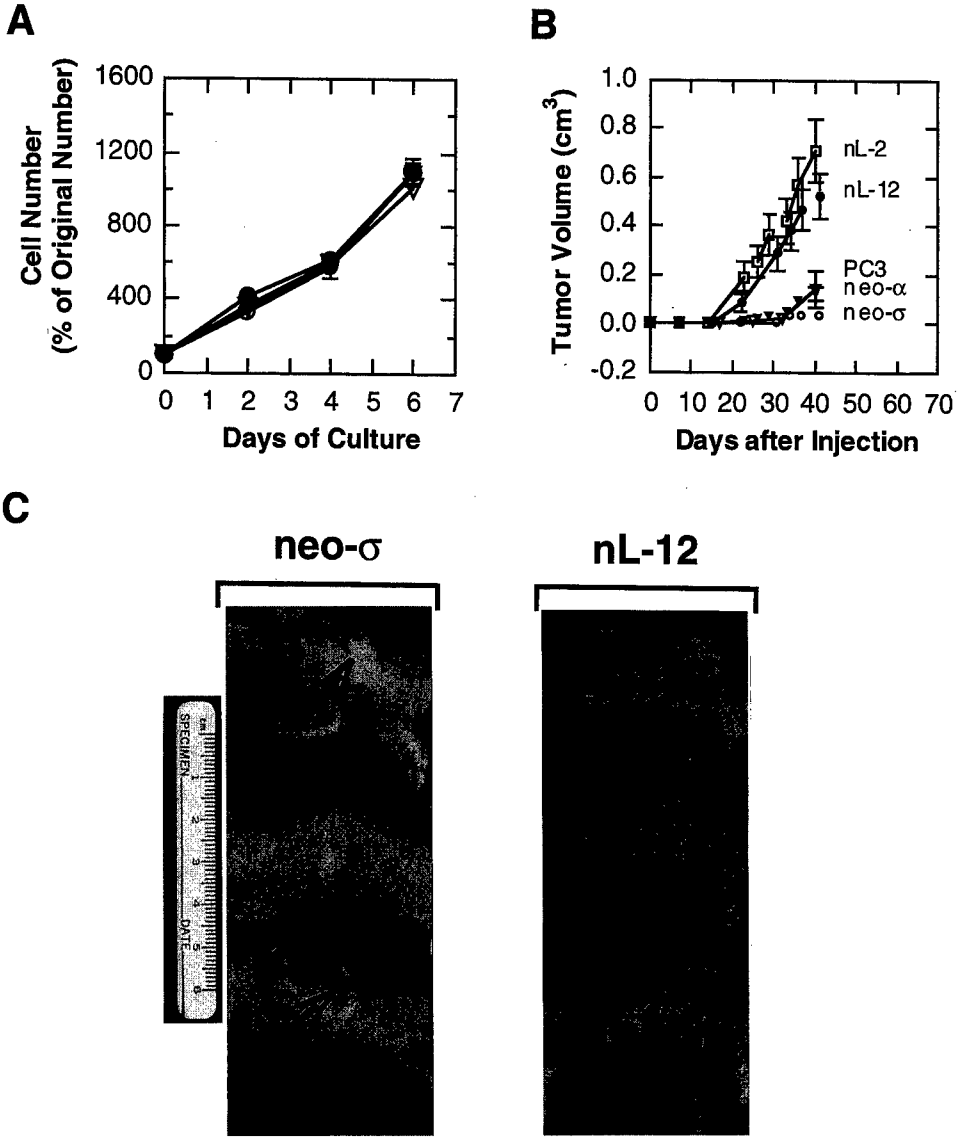

Fig. 2. 12-LOX transfectants have an *in vivo* but not *in vitro* growth advantage. *A*, growth kinetics of PC3 transfectants in culture. Cell proliferation of various transfectants was measured as described in "Materials and Methods." Shown here are the growth curves of PC3 wild type (○), neo-σ (●), nL-8 (▽), and nL-12 (▲). *Data points*, means of six determinations; *bars*, SE. Other clones such as nL-2 and neo-α also had similar growth kinetics (data not shown). *B*, growth kinetics of the tumors derived from 12-LOX transfectants and neo controls. *Data points*, mean volumes of eight tumors for nL-12 (●) and neo-σ (○), five tumors for nL-2 (□) and neo-α (▽), and six tumors for PC3 wild type (▼); *bars*, SE. *C*, mice with tumors from 12-LOX transfectants or from neo control. *Left*, three mice with tumors from neo-σ (*arrows*); *right*, three mice bearing tumors from 12-LOX-transfected PC3 cells (nL-12; *arrows*).

dependent. We found significant vascularization in tumors derived from 12-LOX-transfected PC3 cells, whereas the neo control tumors showed little vessel penetration (Fig. 3*A*). Immunostaining with CD31 antibody, which detects the presence of endothelial cells, showed that the vascular networks in tumors derived from nL-12 were sinusoidal in pattern and well developed in structure (Fig. 3*B*, *right*). In contrast, in neo control tumors, endothelial cells were present but were randomly distributed and did not form an organized vascular network (Fig. 3*B*, *left*). There were fewer vessels in neo-σ tumors than in nL-12, as suggested by microvessel density (Fig. 3*C*). The assessment of the vessel organization demonstrated that the majority of vessels in the tumors derived from 12-LOX-transfected PC3 cells were highly organized, whereas in those from neo-σ, they showed a disorganized to intermediate pattern (Fig. 3*D*). In tumors derived from nL-2 and nL-8, we also observed a similar increase in angiogenesis when compared to neo-α (data not shown).

Increased Angiogenicity of 12-LOX Transfectants. The increased angiogenesis in the tumors generated from 12-LOX-transfected PC3 cells raises the question of whether the observed increase in angiogenesis is the cause or a consequence of the increased tumor growth. To address this issue, we first assayed the conditioned culture medium of PC3 12-LOX-transfected PC3 cells or neo controls for their ability to stimulate endothelial cell migration. As shown in Fig. 4*A*, the medium from the 12-LOX-transfected PC3 cells induced more RV-ECT migration than did the medium from neo controls. Under similar assay conditions, 12(*S*)-HETE itself also stimulated RV-ECT migration at nanomolar levels (Fig. 4*B*). The increased angiogenicity of 12-LOX transfectants was confirmed by the Matrigel implantation assay. As shown in Fig. 4*C*, within 12 days, 12-LOX-transfected PC3 cells (nL-12) in Matrigel induced massive angiogenesis, indicated by the accumulation of blood in the gel, compared to the neo control (neo-σ). The results clearly illustrate that the 12-LOX-transfected PC3 cells are more angiogenic than their neo controls.

Discussion

Here, we found that the increased expression of 12-LOX in human PCa cells stimulates prostate tumor growth by enhancing their angiogenicity. The findings have significant bearing on the regulation of PCa progression because, in patients diagnosed with prostate carcinoma, some tumors are extremely malignant, with rapid progression, whereas others are localized and dormant for many years. Exploration of the mechanism underlying the transition from latent to rapidly growing PCa will provide useful information for PCa management.

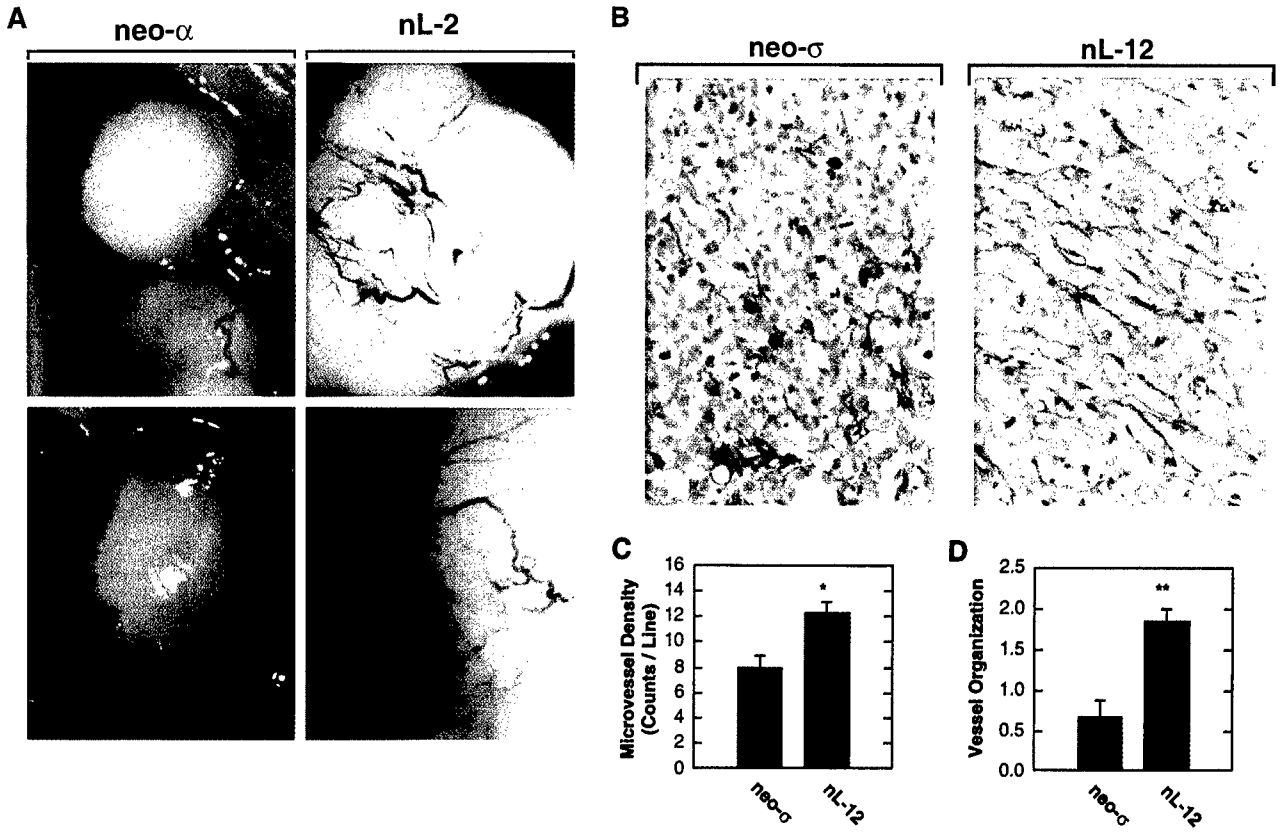

Fig. 3. Increased angiogenesis in the tumors from 12-LOX-transfected PC3 cells. *A*, tumor morphology. *Left*, two tumors from neo-α; *right*, two tumors from 12-LOX-transfected PC3 cells (nL-2). ×8. *B*, CD31 immunostaining. *Brown*, positive staining. *Left*, control tumor. Note the scattered vascular spaces which are randomly distributed and do not form a structured vascular network. *Right*, tumor from 12-LOX-transfected PC3 cells. Note the numerous vascular channels showing a highly organized sinusoidal pattern surrounding small nests of tumor cells. ×250. *C*, microvessel density. *Columns*, microvessel densities, expressed as the average number of vessel-like structures crossing an arbitrary line in one HPF; *bars*, SE. Note the significant increase in microvessel density in the tumors derived from nL-12 ($n = 7$) as compared in those of neo-σ ($n = 7$; *, $P < 0.05$ by Student's t test). *D*, organization of intratumoral blood vessels. The vessel organization was scored as described in "Materials and Methods." *Columns*, mean scores of tumors derived from nL-12 ($n = 7$) and neo-σ ($n = 7$); *bars*, SE (**, $P < 0.01$ by Student's t test).

Our observations here, together with our previous demonstration of the correlation between 12-LOX expression and PCa progression in clinical samples (9), suggest that 12-LOX may play a critical role in the progression of human PCa.

The increased tumor growth observed with 12-LOX-transfected PC3 cells is due to the reduction in tumor necrosis as a result of increased angiogenesis. The increased 12-LOX levels in PC3 cells did not confer a growth advantage *in vitro*, suggesting that 12-LOX overexpression does not have direct effect on PC3 cell growth and that the growth advantage of 12-LOX transfectants *in vivo* is due to the host environment. This tumor-host interaction based mechanism is supported by the observed increase in angiogenesis in the tumors from 12-LOX-transfected PC3 cells. Because angiogenesis is required for tumor expansion, the lack of or inhibition of angiogenesis has been demonstrated to induce tumor cell necrosis and apoptosis, thereby limiting tumor growth in PCa (2, 6–7). Indeed, histological analysis revealed that the tumors derived from neo controls had increased necrosis, suggesting that it is the insufficient vascularization that limited the growth of the neo control tumors.

The increased angiogenesis in the tumors from 12-LOX-transfected PC3 cells is at least partly due to their increased angiogenicity. 12-LOX-transfected PC3 cells have increased ability to stimulate endothelial cell migration *in vitro* and neovascularization of Matrigel *in vivo*, compared to their neo controls. The angiogenicity of tumor cells is controlled by the balance between stimulators and inhibitors of angiogenesis (2). Therefore, it will be interesting to determine how 12-LOX up-regulates the angiogenicity of PCa cells. One explanation is that 12-LOX or 12(*S*)-HETE may increase the angiogenicity of tumor cells by influencing the expression of angiogenic or angiostatic molecules. An alternative interpretation is that 12(*S*)-HETE may directly alter the balance in favor of angiogenic factors due to its proangiogenic nature. This is supported by our finding here that 12(*S*)-HETE stimulated endothelial cell migration at nanomolar levels and previous reports showing that 12(*S*)-HETE stimulated endothelial cell proliferation (14), retraction (15), and adhesion and that it increased the surface expression of integrin $\alpha_v\beta_3$ in both macro- and microvascular endothelial cells (16). It is noteworthy that integrin $\alpha_v\beta_3$ is predominantly associated with angiogenic blood vessels (17) and plays an essential role in human cancer angiogenesis (18). Thus, 12(*S*)-HETE may directly increase the angiogenicity of PCa cells by stimulating angiogenesis or by eliciting several proangiogenic responses that can be additive or synergistic to effects from other angiogenic factors produced by PCa cells because different factors have their own distinct effects on the process of angiogenesis (19). Studies are ongoing to determine whether increased 12-LOX expression in PCa cells influences the gene expression of angiogenic factors and whether 12(*S*)-HETE can stimulate angiogenesis alone or by its additive or synergistic interaction with other putative angiogenic factors.

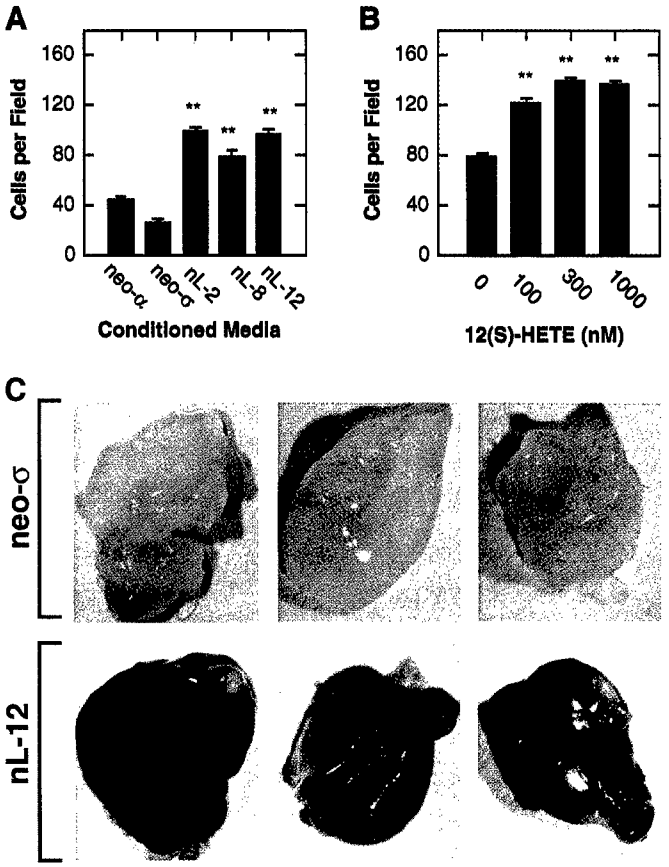

Fig. 4. Increased angiogenicity of 12-LOX-transfected PC3 cells. *A*, stimulation of endothelial cell migration by the conditioned medium from 12-LOX transfectants. The conditioned media were harvested after 24 h of culture and used for migration assay as described in "Materials and Methods." *Columns*, average numbers of cells migrated per field; *bars*, SE (**, $P < 0.01$ by Student's *t* test). *B*, 12(*S*)-HETE stimulates endothelial cell migration. The migration assay was performed essentially as described in *A* except that media with various levels of 12(*S*)-HETE, instead of the conditioned media, were placed into the lower chamber. *Columns*, means; *bars*, SE (**, $P < 0.01$ by Student's *t* test). *C*, induction of angiogenesis in Matrigel by 12-LOX transfectants. *Top*, three Matrigel implants premixed with 2×10^6 neo-σ cells. Note the vessel penetration into the gel was minimal, with little blood accumulated in the gel. *Bottom*, in contrast, the Matrigel premixed with 2×10^6 12-LOX transfectant (nL-12) demonstrates considerable blood accumulation.

One final point concerns the expression of 12-LOX during human PCa progression. If the effects of 12-LOX on PCa tumor growth and angiogenesis just described are of physiological significance, it should be expected that PCa cells express 12-LOX. This has, in fact, been observed *in vivo*, where the expression of 12-LOX has been positively correlated with tumor stage (9). The question of how 12-LOX expression is up-regulated in PCa cells is currently being explored.

Acknowledgments

Special thanks are directed to Dr. Clement Diglio for providing RV-ECT endothelial cells. We thank Dr. Mohit Trikha and Karoly Szekeres for helpful discussions. We acknowledge the excellent technical support from Homan Kian and Ning Wu.

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which demonstrated potent hTrx inhibiton and cytotoxicity, were also screened for their ability to stimulate apoptosis. Based on selection criteria, a number of cytotoxic agents have been identified as potent inhibitors of the Trx system and were assessed for antitumor activity. From the series of active compounds identified, one agent C5, has an IC_{50} for hTrx inhibition of 4 μg/ml and for cytotoxicity of 34 μM, was a potent stimulator of apoptosis and possesses *in vivo* antitumor activity against the MCF-7 and HL60 tumors in scid mice. Supported by the NCIC (DLK) and grants CA48725 and CA77204 (GP).

#836 Novel anti-cancer agents: PS-341, Comparison with new proteasome inhibitors. Elliott, P.J., Lazarus, D.D., Pien, C.S., Palombella, V.J., and Adams, J. *ProScript, Inc., Cambridge, MA 02139.*

Many intracellular proteins are regulated by the ubiquitin-proteasome pathway, including those involved in cell division, tumor growth and metastasis. The ordered and temporal degradation of proteins such as cyclins, cyclin-dependent kinase inhibitors and tumor suppressors are required for cell cycle progression and mitosis. Moreover, activation of the nuclear transcription factor κB is necessary, in part, for the cell viability through the induction of inhibitors of apoptosis. The current studies describe that levels of key proteins are increased, that cells accumulate in the G2-M phase and apoptosis is initiated following inhibition of the proteasome. Moreover, *in vivo* anti-tumor activity is observed following intravenous dose schedules in numerous murine xenograft models. Finally, to determine the pharmacodynamic profile of these proteasome inhibitors, an assay has been developed to follow the inhibition of enzyme activity in blood. Results show that the compounds are potent inhibitors of their target, the proteasome both *in vitro* and *in vivo*. Moreover, the structural differences between the compounds are reflected in their biological activity, including anti-tumor activity. The ongoing clinical development of these novel anti-tumor agents will be discussed.

#837 On the use of three dimensional structural information in the design of potent and selective antagonists of the SH2 domain of Grb2. García-Echeverría, C.,[1] Gay, B.,[1] Rahuel, J.,[2] Caravatti, G.,[1] Fretz, H.,[1] Schoepfer, J.,[1] and Furet, P.[1] [1]*Oncology Research and* [2]*Core Technology, Novartis Pharma Inc., CH-4002 Basel, Switzerland.*

Intracellular signaling pathways that couple growth factor activation with cell control offer new target sites for pharmacological intervention in tumor therapy. This is the case for the protein-protein interactions involving the Src homology 2 (SH2) domain of Grb2 in the Ras signal transduction pathway. Agents that specifically disrupt the interaction between the SH2 domain of Grb2 and its docking site in the activated tyrosine kinase receptors could potentially shut down the Ras pathway and present an intervention point for blocking human malignancy.

The structure of the Grb2-SH2 domain complexed with a phosphotyrosyl peptide was determined in our group by X-ray crystallography. The folding of the SH2 domain of Grb2 shows a general pattern, but the ligand adopts a type I β-turn conformation, in contrast to all previously reported ligand-bound SH2 domain structures where the phosphotyrosyl peptide has an extended conformation. The above unique structural feature has been exploited to increase the *in vitro* potency of the minimal recognition motif of the Grb2-SH2 domain. Molecular modeling selected α,α-disubstituted amino acids were incorporated at the X_{+1} position of Yyy-Tyr(PO_3H_2)-X_{+1}-Asn-NH_2 (Yyy = Ac or 3-amino-Z). These building blocks induce the adoption of a favorable conformation and a boost in the binding affinity for Grb2-SH2. As shown by competitive binding phosphopeptide assays, the structure-based design compounds are not only very potent antagonists but also very selective for Grb2-SH2.

#838 Generation of S. cerevisiae mutants resistant to the angiogenesis inhibitor fumagillin. W-K. Eng, L.F. Faucette, J.L. Raup and R.K. Johnson. *SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406.*

Fumagillin and related compounds have been shown to have potent anti-angiogenic activity. The mechanism of action has been shown to be inhibition of methionine aminopeptidase 2 (MAP2) in mammalian cells. We have confirmed the finding that S. cerevisiae MAP2 is the cellular target of fumagillin. Wild type yeast has two methionine aminopeptidases (MAP1 and MAP2), at least one of which is required for viability. Yeast with functional MAP1 are completely insensitive to fumagillin; deletion of MAP1 renders cells dependent on MAP2 and they become exquisitely sensitive to fumagillin. In order to gain an understanding of the basis for cytotoxicity of fumagillin, we have generated mutants of Δmap1 yeast and selected for resistance to fumagillin. 5 independent experiments generated 14 mutants that were 60–100 fold resistant. PCR cloning and sequencing of the MAP2 gene in these mutants showed that 8 of them had mutations in MAP2. Interestingly, all these mutants demonstrated point mutations at the same residue. Seven had a change of G284D and one had the change G284S. Sequence alignment of the human and yeast MAP2 proteins revealed that the region surrounding Gly284 of yeast was conserved in human. Gly284 of yeast MAP2 aligned with Gly341 of human MAP2. This result indicates that Gly284 is in the site of interaction of MAP2 with fumagillin.

#839 Identification of substrates for membrane type-1 matrix metalloproteinase using bacteriophage peptide display libraries. Ohkubo, S., Miyadera, K., Sugimoto, Y., Matsuo, K., Yoshida, M., Toko, T., and Yamada, Y. *Cancer Research Laboratory, Taiho Pharmaceutical Co., Ltd., 1-27 Misugi-dai, Hanno, Saitama, 357-8527, Japan.*

Membrane type-1 matrix metalloproteinase (MT1-MMP) plays important role in the invasion and metastasis of cancer cells through the activation of proMMP-2. However, there are no sufficient information on substrate specificity of MT1-MMP, although it can digest type I, II, and III collagen. To clarify enzymatic properties of MT1-MMP, we have constructed a random hexamer phage library with a "tether" at the NH_2-terminal of pIII protein and screened for amino acid sequences recognized by this protease. A hexamer of histidine was selected as a tether due to its high affinity to Ni-NTA. The phage library was treated in the solution with human recombinant MT1-MMP, lacking the transmembrane domain, and the cleaved phages were separated from the mixture by a Ni-NTA column. After 4 rounds of selection, the nucleotide sequences of the random region of 15 selected clones were determined and their corresponding amino acid sequences were deduced. Most of selected clones contained proline and leucine which were found to be abundant in collagen. It was suggested that MMPs prefered Pro at the P3 position and Leu at the P1′ position, our experiments provided evidence that these two positions, P3 and P1′, were respectively dominated by Pro and Leu, even in the case of MT1-MMP. Observing the trends in other positions, we found that MT1-MMP prefered Gly or Pro at the P1. These results indicate that the consensus substrate sequence for MT1-MMP is Pro-X-Pro/Gly-Leu at the P3-P1′ sites. This information may have important implication in the design of MT1-MMP inhibitors useful for the development of anti-metastatic agents.

#840 Boronic acid uPA inhibitor that inhibits invasion through reconstituted basement membrane (Matrigel) and cell growth *in vitro*. Schwartz, A.D., Kinder, D.H. *Ohio Northern University, College of Pharmacy, Ada, OH 45810.*

We have been examining certain peptidic boronic acids that are potent and reversible serine protease inhibitors. Recently, we examined the inhibitor Z-Val-Ala-B-Arg (BK-120) as an inhibitor of urokinase plasminogen activator (uPA). uPA has been implicated in both angiogenesis and basement membrane remodeling prior to tumor cell invasion. BK-120 is a weak inhibitor of uPA (K_i = 750 uM against H-Ala-Ala-Pro-Arg-pNA), but showed no inhibition of either model enzymes neutrophil elastase or α-chymotrypsin (bovine). Examination of BK-120's ability to inhibit cell growth in the HT-29 human colon tumor cell line using the MTT assay consistently gave an IC_{50} of about 30 μM. Colony formation assay using compound concentrations around the 30 μM level resulted in a 10 – 20% reduction in number of colonies relative to vehicle control. Expression of the angiogenic factor basic FGF was suppressed to 15% of control at BK-120 concentrations of 5 μM. We examined BK-120's ability to prevent tumor cell invasion through reconstituted basement membrane (Matrigel), and found that at concentrations that inhibited growth by 50% in the MTT assay (but which were not appreciably cytotoxic), the number of cells that invaded the membrane could be completely blocked. Thus, our preliminary results support a role for uPA in cell growth and invasion in the HT-29 tumor cell line that may be related to expression of the angiogenic factor basic FGF. uPA therefore may be an excellent target for further chemotherapeutic agent development.

#841 Inhibition of prostate cancer cells by a novel 12-lipoxygenase inhibitor in a human "orthotopic bone metastasis" model. Nie, D., Nemeth, J.A., Cher, M.L., Chen, Y., Barroso, U., and Honn, K.V. *Wayne State University, Detroit, MI 48201.*

Expression of the enzyme 12-lipoxygenase (LOX) and its product 12(S)-HETE are associated with human prostate cancer progression and metastasis, and evidence has accumulated that 12(S)-HETE is an intracellular signaling molecule which results in tumor angiogenesis. A drug screening program resulted in the identification of a novel compound, HA188, which is a relatively non-toxic, potent inhibitor of 12-LOX. We wished to test the ability of this drug to alter the growth prostate cancer cells in the human bone microenvironment. We generated stable PC-3 transfectants which produce high levels of 12-LOX and 12(S)-HETE. SCID-human-bone mice were created by subcutaneous implantation of macroscopic human fetal femur fragments in SCID mice. After four weeks, these bone fragments were directly injected with 10^5 PC-3 cells which were over expressing 12-LOX. Large bone tumors developed rapidly, characteristic of the SCID-human prostate cancer "orthotopic bone metastasis" model. Mice with tumors of similar size were treated every other day with intraperitoneal injections of high, low, and vehicle-control doses of HA188. We found that HA188 inhibited bone tumor growth in a dose-dependent fashion. A dose of 100 mg/kg inhibited tumor growth 6-fold compared with control. Histological analysis showed tumor necrosis. These results show that selective inhibition of 12-LOX retards prostate cancer bone tumor growth of 12-LOX-transfected prostate cancer cells in the scid-human "orthotopic bone metastasis" model.

#842 Inhibition of human prostate cancer (HPC) growth by vitamin-D analogues (VDA) is attenuated by increases in stromal and extracellular matrix (ECM) interaction. Sokoloff, MH, Caven, TH, Chang, S-M, Wood, AW, and Chung, LWK. *University of Virginia, Charlottesville, VA 22901, and Roche Pharmaceuticals, Nutley, NJ 07110.*

nocytes when stimulated by the invasion-promoting growth factor, HGF. The role of activated ERK in stimulating both MMP9 production and cell motility was assessed by suppressing ERK activation in a dose-dependent manner using MEK specific inhibitor, PD98059, in HGF-stimulated keratinocytes. While levels of activated JNK and levels of activated c-Jun transcription factor remained unchanged, ERK activation was suppressed by PD98059 and this was accompanied by a dose-dependent inhibition of cell motility and MMP9 production and by a decline in c-Fos steady state levels. Furthermore, ERK-dependent cell motility involved activation of the *m*yosin *l*ight chain *k*inase (MLCK), whose activity is essential in generating contractile forces for cell motility. When JNK activity was suppressed by transfection of HGF-stimulated keratinocytes with a dominant interfering expression vector, JNK(APF), MMP9 production and AP-1 activity was suppressed but motility of JNK(APF) expressing cells was not inhibited. These results suggest that the increase in MMP9 production and in the activation of the AP-1 complex is directly dependent upon coordinate activation of ERK and JNK activity. However, the ERK pathway, but not the JNK pathway, is both necessary and sufficient for stimulation of cell motility by a mechanism requiring activation of MLCK.

#1314 Tumor dormancy due to urokinase receptor (uPAR) downregulation is dependent on $\alpha5\beta1$ signaling and activation of p42/44 ERK *in vitro* and *in vivo*. Aguirre-Ghiso, J.A., Liu, D., Ossowski, L. *Div. of Neoplastic Diseases, Department of Medicine, Mount Sinai School of Medicine, New York, NY10029.*

Mechanisms regulating tumor dormancy remain relatively unexplored. We have previously shown that uPAR downregulation forces tumor cells into a dormant state due to a reduction in proliferation. We have now analyzed the mechanisms through which signaling may be involved in regulating this process *in vivo* and *in vitro*. Human Hep3 cells stably transfected with an antisense for human uPAR (AS24:dormant cells) or with an empty vector (LK25:tumorigenic cells) or non-transfected (T-Hep3:parental tumorigenic cells) were employed. Upon inoculation on the chick embryo CAM only AS24 cells arrested in G0/G1, while T-Hep3 cells continued to proliferate. In culture AS24 cells had almost undetectable basal levels of active phospho-ERK1/2 while LK25 or T-Hep3 cells have highly activated ERK1/2. The MEK-1 inhibitor PD098059 inhibited this constitutive activation. Dose-response assays showed that ERK1/2 could be readily activated by 1–10 nM scuPA at 5 min. in LK25 or T-Hep3 cells while ~30 fold more scuPA was required to marginally activate ERK1/2 in AS24 cells. This activation was also blocked by PD098059. Time course studies showed that activation of ERK1/2 by scuPA was near maximal at 5 min while AS24 displayed marginal activation even at 30 min. Since uPAR may interact with $\beta1$-integrins we studied the modulation of integrin function in tumorigenic or dormant cells. AS24 cells displayed a lower adhesion to fibronectin (FN) compared to LK25 or T-Hep3 cells, which was not due to reduced $\alpha5\beta1$ expression. Impaired adhesion of AS24 cells was restored by $\beta1$ activating antibodies (TS2/16) or by addition of $MnCl_2$. FN stimulated LK25 or T-Hep3 cells growth in culture and ERK1/2 activation while only marginal effects were observed on AS24 cells. These results suggest that uPAR down-regulation blocks uPA:uPAR and $\alpha5\beta1$-integrin dependent activation of the MEK1-ERK1/2 pathway rendering tumorigenic cells dormant *in vivo*.

#1315 AP-2 expression reduces the metastatic potential of MDA-MB-231 breast cancer cells. Van Laar, E.S., Bar-Eli, M., Price, J.E. *University of Texas M. D. Anderson Cancer Center, Houston, TX 77030.*

The transcription factor AP-2 regulates gene expression in epithelial and neural crest-derived cells. Loss of AP-2 expression has previously been correlated with the metastatic phenotype of melanoma cells. Comparing expression in a panel of human breast cancer cell lines, the MDA-MB-231 line showed low levels of AP-2, detected by RT-PCR. This cell line was transfected with an AP-2 expression vector, and clones isolated. Analyses of nuclear extracts from the transfected clones revealed proteins that bound to AP-2 binding motifs in EMSA gels, and that showed a supershift in the presence of AP-2 specific antibodies. The AP-2-expressing MDA-MB-231 cells showed slower growth in monolayer culture and reduced colony forming efficiency (40 – 50% reduction) when grown in semi-solid agarose cultures. Preliminary *in vivo* experiments showed that the AP-2-transfected cells produced fewer experimental lung metastases than the control cells when injected i.v. into nude mice. However, tumorigenicity and growth in the mammary fatpad were not altered by introduction of the transcription factor. The results implicate AP-2 in the regulation of genes controlling the metastatic potential of MDA-MB-231 human breast cancer cells.

#1316 A single nucleotide polymorphism (SNP) in the matrix metalloproteinase-1 (MMP-1) promoter is a marker on 11q22 for loss of heterozygosity (LOH) and may influence tumor progression. Rutter, J.L., Fleming, E.L., Ernstoff, M., Coon, C.I., Klein, C., Ozelius, L.J., and Brinckerhoff, C.E. *Dartmouth Medical School, Hanover, NH 03755 and Massachusetts General Hospital and Harvard Medical School, Charlestown, MA 02129.*

We have isolated a SNP in the promoter of MMP-1, collagenase-1, which is implicated in tumor cell invasion/metastasis due to its ability to cleave the interstitial collagens types I and III. Since this SNP may influence the pathological expression of MMP-1, understanding its role in cancer progression is important. We cloned and sequenced over 4 kb of the endogenous MMP-1 promoter from the aggressive A2058 melanoma tumor cells and compared the sequence to a previously isolated clone derived from a human leukocyte genomic library. The promoter from the A2058 cells contained an extra guanine base (G) at pc −1607 bp, changing the sequence from (5'-AAGAT-3') to (5'-AAGGAT-3'), the 2G variation creates an Ets transcription factor binding site (EBS). The M EBS-SNP increased transcription in various tumor cell lines, and is a true morphism, not a mutation. To test whether the frequency of the EBS-S increased in tumors, we examined 7 breast cancer cell lines and 44 mela tumors for this variation by PCR. The frequency of the EBS-SNP in contro is 30%, but increased to 72% in the cell lines (P=0.0001), and to 43% (P= in the patient tumors. Of the 44 patient samples, 7 had normal tissue counte and were studied for LOH. Of the 7 cases, 83% (5/6) of the tumors contai 2G allele displayed LOH at this site. Using the tumor DNA, we identif breakpoint at 11q22 between markers D11S2002 and D11S4464, the r where MMP-1 resides. Thus, our data suggest that MMP-1 plays a role in progression.

#1317 Arachidonate 12-lipoxygenase enhances the metastatic pot of human prostate cancer cells. Nie, D., Chen, Y., Tang, K., Hanna, K., Ne J., Hillman, G.G., Cher, M., Grignon, D., and Honn, K.V. *Wayne State Univ Detroit, MI 48202.*

We have previously found a positive correlation between the expressi arachidonate 12-lipoxygenase (LOX) and tumor stage in human prostate noma (PCa; Gao *et al.*, 1995. Urology *46*: 227–237) and that platelet-type 1 stimulated PCa tumor growth by increasing the angiogenicity of cancer cell *et al.*, 1998. Cancer Res. *58*: 4047–4051). This study examines whethe lipoxygenase also modulates the metastatic potential of human PCa cells cells stable transfected with 12-LOX cDNA expression construct are more sive toward vitronectin, type I and IV collagens, but not to fibronectin or la when compared with mock controls or PC3 wild type. 12-LOX transfecte cells also have increased haptotactic motility to vitronectin but not to fibron Tumor cell invasion to local muscle or fat tissues and lymph node enlargeme more frequent in nude mice bearing s.c. tumors from 12-LOX transfecte cells than in those with the mock controls. When injected via tail vein into mice with s.c. implanted human bone and lung fragments, increased freque macrometastasis in human bone implants, human lung implants, lymph and mouse lung are observed in PC3 cells with high 12-LOX levels. Ou suggest 12-LOX promotes the metastatic potential of human PCa cells.

#1318 IL-10/IL-10 receptor activation of a 'LIM-only' signal mol regulating tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) expre and metastasis. Wang Min, Youji Hu and Stearns Mark. *Department of P ogy, Medical College of Pennsylvania and Hahnemann University, Schc Medicine, Philadelphia, PA 19102.*

We shown that the IL-10/IL 10R axis activates a 5' promoter enhancer ele (28 bp) of the TIMP-1 gene to stimulate a ~3–7 fold increase in TIMP-1 mRN protein production by 24 hr in human prostate PC-3 ML cells (Wang et al., C Res. 10, 219, 1998). Recently, we have characterized the IL-10/IL-10R axis pathway and found that IL-10 activates tyrosine phosphorylation and rapi clear translocation of a 23 Kda cysteine rich transacting factor (CRTF induces enhancer dependent TIMP-1 expression. We have cloned the CRTF and raised polyclonal antibodies against peptide sequences. Immunopreci combined with Western blotting demonstrated that CRTF was tyrosine phos ylated after treating the cells with IL-10 for 10–60 min. Gel antibody supe assays confirmed that CRTF specifically binds the TIMP-1 promoter enh element. Electrophoretic gel migration shift assays showed that CRTF wa nificantly over expressed in prostate cancer gland nuclear extracts an expressed in HGPIN or benign prostate glands, indicating CRTF might marker for prostate cancer. Finally, IL-10 induced TIMP-1 expression to tumor growth and metastasis in human PC-3 ML orthotopic tumors in SCID The data indicate IL-10 might be therapeutic in preventing tumor metas

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RADIOBIOLOGY/RADIATION ONCOLOGY 2: Molecular Pathways of Cellular Radioresponse

#1319 Characterization of ATM kinase substrate specificity. Lim, Kim, S-T., Canman, C.E., and Kastan, M.B. *Department of Hematology-Onc St. Jude Children's Research Hospital Memphis, TN 38105-2794 USA.*

Ataxia telangiectasia (AT) is a rare human autosomal recessive disease pleiotropic phenotypes characterized by neuronal degeneration, immune function, hypersensitivity to ionizing radiation and cancer predisposition gene mutated in A-T, ATM (ataxia telangiectasia-mutated), encodes a 3 proteins that bears homology to a family of protein kinases related to pho tidylinositol 3-kinase. We have recently shown that recombinant ATM k phosphorylates p53 on Ser15 *in vitro*, a site that is also phosphorylated *in v* response to ionizing radiation. Ionizing radiation enhances endogenous kinase activity, suggesting that ATM acts upstream of p53 in the DNA dan induced signal transduction pathway. Here, we demonstrate that ATM k

PROSTAGLANDINS & OTHER LIPID MEDIATORS
(in press)

ARACHIDONATE 12-LIPOXYGENASE STIMULATES TUMOR ANGIOGENESIS BY UP-REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION

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Platelet-type 12-lipoxygenase (LOX) was previously shown to stimulate prostate tumor growth which was closely related with increased tumor angiongenesis. In this follow-up study, we investigated whether 12-LOX can stimulate the expression of putative angiogenic factors that might contribute to the increased tumor angiogenesis. Increased expression of vascular endothelial growth factor (VEGF), but not basic fibroblast growth factor (bFGF) or interleukin 8 (IL-8), was detected in 12-LOX transfected PC3 cells as compared to vector-controls. Northern Blot analysis found there were increased 12-LOX mRNA levels in 12-LOX transfected PC3 cells. Inhibition of 12-LOX activity with either general LOX inhibitor nordihydroguaiaretic acid (NDGA) or specific 12-LOX transfected PC3 cells. Neutralization of VEGF by a function blocking antibody abolished the ability of 12-LOX transfected PC3 cells to stimulate endothelial cell migration. Our data suggest 12-LOX regulate tumor angiogenesis by stimulating VEGF expression.

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